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Cyclin E is an unstable protein that is degraded in a ubiquitin- and proteasome- dependent pathway. Two factors stimulate cyclin E ubiquitination *in vivo*: when it is free of its CDK partner, and when it is phosphorylated on threonine 380. We pursued the first of these pathways by using a two-hybrid screen to identify proteins that could bind only to free cyclin E. This resulted in the isolation of human Cul-3, a member of the cullin family of E3 ubiquitin-protein ligases. We found that Cul-3 was bound to cyclin E but not to cyclin E-Cdk2 complexes in mammalian cells, and that overexpression of Cul-3 increased ubiquitination of cyclin E but not other cyclins. Conversely, deletion of the *Cul-3* gene in mice caused increased accumulation of cyclin E protein, and had cell-type specific effects on S phase regulation. In the extraembryonic ectoderm, where cells undergo a standard mitotic cycle, there was a greatly increased number of cells in S phase. In the trophectoderm, where cells go through endocycles, there was a block to entry into S phase.

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Jeffrey D. Singer 10/2/01

PI - Signature Date

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INTRODUCTION

Cyclins have been shown to be proteins that both activate cyclin-dependent kinases and impart some degree of substrate specificity to the cyclin-CDK holoenzyme. Although very little is known about what these substrates are, it is clear that the regulation of levels of cyclins is crucial for cell cycle progression. In the yeast *Saccharomyces cerevisiae* several cyclins have been identified. Some, the CLNs, appear to be specific for the G1/S transition while others, the CLBs appear to be specific for the G2/M boundary. The human cyclin E gene was cloned based on its ability to complement a yeast strain with mutations in its G1 cyclin genes CLN1-3. The cyclin E protein has subsequently been shown to be required for the G1/S transition in mammalian cells. Protein levels have been shown to cycle as the cell cycle progresses such that levels of cyclin E are maximal in early S phase and then rapidly decline. The goal of the work proposed here is to identify the components that are responsible for the regulation of the cyclin E protein levels. Such regulation has many important implications for the study of cell cycle progression and thus the onset of tumorigenesis which is an example of uncontrolled cell cycle progression. Recently, elevated levels of cyclin E protein have been correlated with severity of breast cancer.

BODY

The following three aims were approved in the Statement of Work:

Specific Aim 1) Are proteins that physically interact with cyclin E protein involved in its degradation?

Specific Aim 2) Can an *in vitro* system for regulation of cyclin E be developed by identifying the proteins that regulate ubiquitination of cyclin E?

Specific Aim 3) Are the human homologues of the proteins that regulate yeast G1 cyclins responsible for the degradation of human cyclin E?

The first aim has been addressed during the first year of support. A yeast 2 hybrid screen was utilized to identify proteins that bind cyclin E. The results of the two hybrid screen and subsequent analysis of the protein that was identified has been published by the journal Genes and Development and appeared in the September 15 issue.

KEY RESEARCH ACCOMPLISHMENTS (First year)

- Identification of a cyclin E binding protein that is homologous to known cyclin degradation proteins
- Cloning of the cul-3 gene and development of reagents to study its role in the degradation of cyclin E in cells. These reagents include antibodies, cells lines, expression vectors and knock out mice.
- Extensive analysis of knock out mice (still under way).

The second aim was addressed during the second year of support. Baculo expressed cyclin E bound to its kinase CDK2 were made and the requirements for phosphorylation of cyclin E and disassociation of the complex were studied. In addition further analysis of the cul-3 protein were performed by the construction of a conditional knockout of the cul-3 gene. During the analysis of cul-3 other binding proteins were identified which led to an analysis of cul-3 binding to the CDK2 inhibitor p21. These data were published in Molecular Cell in February 2000.

KEY RESEARCH ACCOMPLISHMENTS (Second year)
- Construction of a conditional knockout of the cul-3 gene in mice
- 2-hybrid screens using the cul-3 gene as bait to identify components of the degradation complex
- development of a *in vitro* system to study the cyclin E-CDK2 interaction

The research undertaken during the third year of support involved analysis of the role of phosphorylation on the degradation of human cyclin E. We were able to show that a crucial threonine, T380, was phosphorylated by the kinase GSK3β. This phosphorylation had previously been proposed to be the signal for degradation, however we have shown that in fact it regulates binding of cyclin E to its cognate kinase CDK2. These data are presently being written as a paper.

KEY RESEARCH ACCOMPLISHMENTS (Final year)
- Development of a phosphorylation specific antibody that recognized only threonine 380 on cyclin E when it was phosphorylated.
- Identification of the kinase, GSK3β, that phosphorylated cyclin E on threonine 380.
- Continued breeding of the cul-3 conditional knockout to create an F1 generation.

REPORTABLE OUTCOMES

•Part of the data obtained during the final year of support will be submitted for publication this year (journal as yet to be determined).

•I have accepted a faculty position, effective September 1 2001, as Assistant Professor in the Department of Molecular Biology, Cell Biology and Biochemistry at Brown University in Providence, Rhode Island.

CONCLUSIONS

Based on what we have learned so far about the role of cul-3 in the degradation of cyclin E in mammalian cells we are pursuing several studies to determine if cul-3 can prevent unwanted cell growth in normal organs. In addition we will use the antibodies we have developed that recognize cul-3 to analyze breast tumors samples for misregulation of cul-3 protein. I now have a conditional knock out of cul-3 in mice that I will use to generate cells lacking cul-3. In addition these mice can be used to study development in which cul-3 is absent.

APPENDICES

The two published papers accepted during the funding period.

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Jeffrey D. Singer, Ph.D.

Cullin-3 targets cyclin E for ubiquitination and controls S phase in mammalian cells

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Cyclin E is an unstable protein that is degraded in a ubiquitin- and proteasome- dependent pathway. Two factors stimulate cyclin E ubiquitination in vivo: when it is free of its CDK partner, and when it is phosphorylated on threonine 380. We pursued the first of these pathways by using a two-hybrid screen to identify proteins that could bind only to free cyclin E. This resulted in the isolation of human Cul-3, a member of the cullin family of E3 ubiquitin–protein ligases. We found that Cul-3 was bound to cyclin E but not to cyclin E-Cdk2 complexes in mammalian cells, and that overexpression of Cul-3 increased ubiquitination of cyclin E but not other cyclins. Conversely, deletion of the *Cul-3* gene in mice caused increased accumulation of cyclin E protein, and had cell-type-specific effects on S-phase regulation. In the extraembryonic ectoderm, in which cells undergo a standard mitotic cycle, there was a greatly increased number of cells in S phase. In the trophectoderm, in which cells go through endocycles, there was a block to entry into S phase. The SCF pathway, which targets cyclins for ubiquitination on the basis of their phosphorylation state, and the Cul-3 pathway, which selects cyclin E for ubiquitination on the basis of its assembly into CDK complexes, may be complementary ways to control cyclin abundance.

[*Key Words*: Cullins; ubiquitin degradation; cyclins; S phase]

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Cyclin E is an evolutionarily conserved protein whose essential function is to promote the cell cycle transition from G_1 to S phase (Knoblich et al. 1994; Ohtsubo et al. 1995). Cyclin E binds to and activates the cyclin-dependent kinase Cdk2, and it is the catalytic activity of this holoenzyme that mediates the effects of cyclin E on cell cycle (Fang and Newport 1991; Koff et al. 1991, 1992, 1993; Dulic et al. 1992; Jackson et al. 1995). Hence, mutants of cyclin E that cannot bind to CDK2 are biologically inert (Kelly et al. 1998), and ectopic overexpression of a catalytically inactive mutant of CDK2 prevents a cell from entering S phase (Heuvel and Harlow 1993). Conversely, elevated amounts of active cyclin E–CDK2 accelerate entry into S phase (Ohtsubo and Roberts 1993; Resnitzky and Reed 1995). The substrates of cyclin E–Cdk2 are not fully defined, but are thought to include inhibitors of S phase such as pRb (Hinds et al. 1992; Connell-Crowley et al. 1997; Zarkowska and Mittnacht 1997; Kelly et al. 1998) and p27Kip1 (Sheaff et al. 1997), which are inactivated by CDK2-directed phosphorylation. Additionally, proteins that stimulate DNA synthesis might be phosphorylated and thereby activated by CDK2 (Blow and Nurse 1990; D'Urso et al. 1990; Zhao et al. 1998).

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The abundance of the cyclin E protein and cyclin E–Cdk2 catalytic activity oscillate in parallel during the cell cycle, reaching a peak as cells begin chromosome replication and a trough at G_2/M (Koff et al. 1991; Dulic et al. 1992; Ohtsubo et al. 1995). This is achieved, in part, by cell cycle-dependent gene transcription (Ohtani et al. 1995; Geng et al. 1996). The cyclin E promoter contains E2F-binding sites, and activation of the E2F transcriptional program during G_1 increases cyclin E gene expression. Phosphorylation of the Retinoblastoma (Rb) protein by the cyclin D-associated kinases is initially responsible for the release of E2F and increased expression of E2F-responsive genes (Weinberg 1995). Hence, the D-type cyclins function upstream of cyclin E in a pathway that leads to cyclin E gene expression (Lukas et al. 1995; Geng et al. 1999; Leng et al. 1997). Expression and activation of cyclin D–CDK complexes is positively controlled by mitogens, which indirectly establishes the dependency of cyclin E accumulation on mitogenic signaling (Sherr 1995). Therefore, increased expression of cyclin E in pre-S-phase cells reflects the fact that the mitogen requirement for cell proliferation has been satisfied and also represents a necessary step for beginning DNA synthesis.

A second essential component of cyclin E periodicity is post-transcriptional regulation by ubiquitin-dependent proteolysis (Clurman et al. 1996; Won and Reed

1996). Cyclin E has a short half life of less than 30 min, which can be increased to greater than 2 hr by pharmacologic inhibition of the proteasome (Clurman et al. 1996; Won and Reed 1996). Rapid turnover of cyclin E protein ensures that its levels closely parallel the changing abundance of its mRNA, and therefore underlies the strict dependence of cyclin E protein expression on the cyclin D/Rb/E2F pathway. Turnover of cyclin E by the ubiquitin–proteasome pathway is regulated both by the binding of cyclin E to CDK2, and by cyclin E phosphorylation (Clurman et al. 1996; Won and Reed 1996). Thus, unbound cyclin E is readily ubiquitinated and degraded by the proteasome, whereas cyclin E within cyclin E–CDK2 complexes is protected from ubiquitination. However, the protection afforded by CDK2 is reversed in a process that involves phosphorylation of cyclin E on threonine 380, which triggers ubiquitination of cyclin E and degradation of cyclin E in the proteasome.

The regular rise and fall of cyclin E protein levels is an essential feature of normal cell cycle regulation. Firstly, the upswing in cyclin E expression is one means by which exit from G_1 is coupled to the receipt of extracellular mitogenic cues or other proliferative signals. Secondly, the timing of S phase is determined by the abundance of the cyclin E protein. Thirdly, the decline in cyclin E abundance later in S and G_2 resets the cell cycle program to its initial state, and thereby reestablishes the dependency of G_1 progression on mitogens in the ensuing cell cycle. Finally, cyclin E oscillation appears to be essential for endocycles, cell cycles in which sequential S phases occur without intervening mitoses. It is thought that each cycle of chromosome replication must be accompanied by a decrease and then a rise in cyclin E activity (Folette et al. 1998; Weiss et al. 1998).

The proteins that select cyclin E for ubiquitination are not known. Identifying these proteins will be essential for understanding how cyclin E is recognized by the ubiquitin–proteasome pathway, for determining whether the activities of the relevant ubiquitinating enzymes are regulated during the cell cycle or modulated by extracellular signals, and for addressing whether the pathways responsible for cyclin E turnover are altered in tumorigenic cells that display deregulated cyclin E expression. Using a combination of molecular and genetic approaches, we have identified a member of the cullin family, Cul-3, as being one component of a pathway that controls cyclin E ubiquitination. Homozygous deletion of the *Cul-3* gene is shown to cause overexpression of the cyclin E protein and to disrupt normal cell cycle regulation in vivo.

Results

Cloning and characterization of human Cullin-3

Our previous work indicated that one pathway for ubiquitination of cyclin E was critically affected by the binding of cyclin E to a CDK (Clurman et al. 1996). Thus, free (unbound) cyclin E was readily ubiquitinated, whereas cyclin E bound to a CDK was protected from ubiquitination. To identify molecules that might be involved in targeting free cyclin E for ubiquitination, we performed a two-hybrid screen in which a mutant version of cyclin E (cyclin E R130A) was used as bait. In both mammalian cells and yeast wild type, cyclin E binds to and activates CDKs, whereas cyclin E(R130A) cannot. Clones that scored positively for an interaction with cyclin E(R130A) were rescreened against wild-type cyclin E. From 1.5×10^6 transformants we identified a single protein that was able to bind to cyclin E R130A but could not bind to wild-type cyclin E (Fig. 1A), properties that were consistent with it having a role in targeting cyclin E for ubiquitination. The DNA sequence of this interactor revealed that it was a portion of the protein Cullin-3 (Cul-3) (amino acids 395–768). These binding properties were not an artifact of using a truncated Cul-3 protein, because reconstruction experiments demonstrated that full-length Cul-3 also bound to cyclin E R130A, and not to wild-type cyclin E in this assay (not shown). Cul-3 is a member of the cullin family of genes, defined as homologs of the *Cul-1* gene from nematodes (Kipreos et al. 1996; Du et al. 1998). This family includes the Cdc53 protein in budding yeast, which has been shown to be part of an E3 ubiquitin–protein ligase (Patton et al. 1998).

The partial Cul-3 clone obtained in the two-hybrid screen was used as a probe to isolate cDNAs from a human B-cell library containing the complete Cul-3 ORF. The complete Cul-3 ORF is 2307 bp and is predicted to

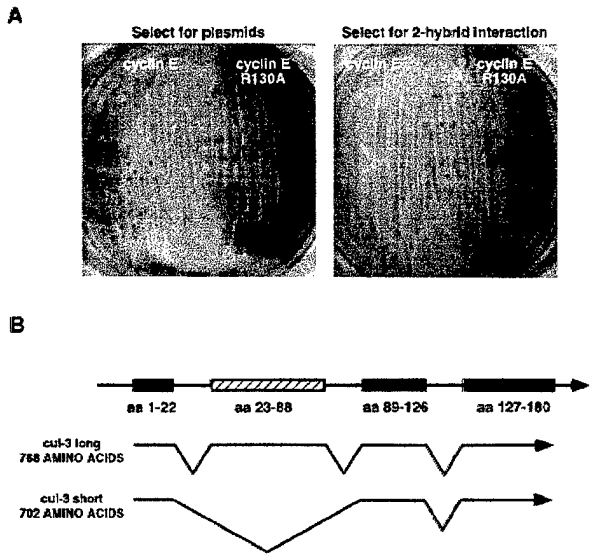

Figure 1. Cloning of human Cullin 3. (*A*) Two-hybrid interaction between cyclin E and Cul-3. (*Left*) Growth of *Saccharomyces cerevisae* when selected for the presence of the cyclin (either wild-type cyclin E or a CDK nonbinding mutant) and cullin plasmids; (*right*) growth of same strains when selection is applied for a two-hyrid interaction between the cyclin and the cullin. (*B*) Graphical representation of the two Cul-3 cDNAs identified. Cul-3 long contains 768 amino acids and Cul-3 short is missing one exon of 66 amino acids and is therefore only 702 amino acids long.

encode a protein of 768 amino acids. The amino acid sequence shows homology to all the cullins in the databases with the greatest similarity in the cullin domain, which includes amino acids 741–768 in the Cul-3 sequence. Cul-3, like other cullins, lacks a HECT domain, a sequence found in a subset of E3 enzymes.

A second cDNA also apparently containing a complete Cul-3 ORF was isolated in this same screen. However, this clone encoded a version of Cul-3 with an in-frame deletion of amino acid 23 through amino acid 88 (Fig. 1B). Subsequent sequencing of the *Cul-3* gene (not shown) revealed that the deleted region precisely corresponded to an exon, and that the two cDNAs (hereafter referred to as Cul-3 long and Cul-3 short) therefore represented alternatively spliced forms of Cul-3 mRNA.

Pattern of Cul-3 protein expression

Portions of Cul-3 corresponding to the amino, middle, and carboxy parts of the protein were individually expressed as recombinant proteins in *Escherichia coli* and used separately to immunize rabbits, thereby generating three distinct antisera that recognize different regions of the Cul-3 protein (see Materials and Methods). All three antisera were affinity purified against the immunizing antigen and when used for immunoblotting of whole cell extracts they were found to recognize a single protein with the predicted molecular size of Cul-3 (Fig. 2; data not shown). Each of the antibodies detected increased expression of full-length Cul-3 protein in whole cell extracts from mammalian cells that had been transfected with a CMV promoter-driven mammalian expression vector containing the Cul-3 cDNA, and none of the antibodies recognized Cul-1. Both the transfected and endogenous protein run as a doublet in all cells examined (see below). Finally, no immunostaining was detected in *Cul-3*$^{-/-}$ mice (see Fig. 5, below), confirming the specificity of the antibodies for Cul-3.

Various cell types, both mortal and immortal, from mice and humans, were stained with each of the Cul-3 antibodies to determine the subcellular location of the Cul-3 protein (see Materials and Methods for complete listing of all cell types examined). All three antibodies, and all of the cell types we examined, demonstrated the same pattern of Cul-3 localization to both the nucleus and the Golgi (Fig. 2B). Golgi staining was confirmed by use of rhodamine-tagged wheat germ agglutinin to visualize the Golgi, and by specific dissolution of the Golgi

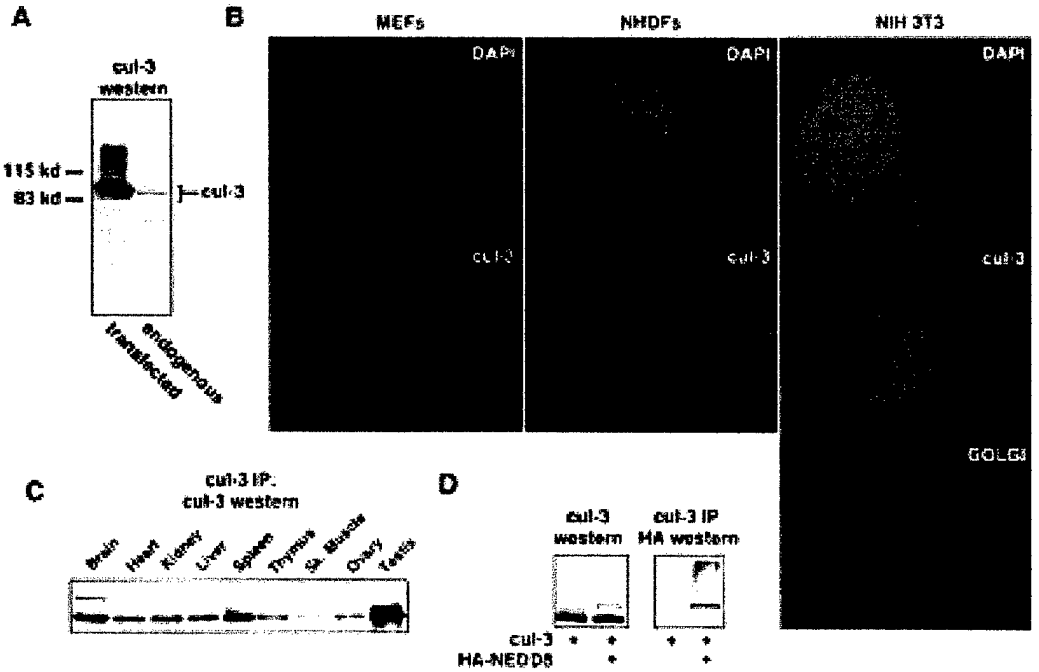

Figure 2. Characterization of human Cul-3. (*A*) Immunoblot of Cul-3 in whole cell extracts from h293 cells. (*Left*) Extract from cells transfected with full-length Cul-3 cDNA; (*right*) untransfected cell extract. (*B*) Immunolocalization of Cul-3. The indicated cells were stained with DAPI and Cul-3 antibodies. In addition, the NIH-3T3 cells were stained with rhodamine-conjugated wheat germ agglutinin to identify the Golgi apparatus. (*C*) Cul-3 expression in mouse tissues. Cul-3 was immunoprecipitated from tissue extracts with antibodies against the carboxy-terminal portion of Cul-3,and then analyzed by Western blots with antibodies against the amino-terminal portion of Cul-3. (*D*) NEDD8 modification of Cul-3 protein. (*Left*) Cul-3 was transfected into h293 cells with or without HA-NEDD8. Extracts were blotted with anti-Cul-3 antibodies. Inclusion of epitope-tagged NEDD8 shifted the mobility of higher molecular weight form of Cul-3. (*Right*) Cul-3 protein was immunoprecipitated from the same extracts and the material was analyzed on a Western blot with anti-HA antibody to detect NEDD8. The higher molecular weight form of Cul-3 was immunoreactive with the anti-HA antibody.

with Brefelden A (not shown). The Golgi localization of Cul-3 was more dramatic in murine compared with human cells, but this appeared to represent a difference in the elaboration of the Golgi in mouse versus human cells rather than a difference in Cul-3 itself.

In asynchronously proliferating cells there was no obvious cell to cell heterogeneity in the Cul-3 staining pattern that would suggest its distribution changed during the cell cycle; the one exception being the pancellular distribution seen in mitotic cells (not shown). Also, MANCA cells were separated according to position in the cell cycle by centrifugal elutriation and whole cell extracts immunoblotted for Cul-3 protein. This revealed no cell cycle-dependent changes in Cul-3 protein expression (not shown).

These same antibodies were used to determine the tissue distribution of the Cul-3 protein in adult mice. As shown in Figure 2C, Cul-3 protein is expressed in all tissues examined with the greatest amount of Cul-3 expressed in brain, spleen, and testis. Cul-3 expression was also detected in all cell lines examined, and its abundance did not differ substantially between primary and immortalized cell lines (data not shown).

Cul-3 is modified by NEDD8

Mammalian cullins (Osaka et al. 1998; Wada et al. 1999) and the yeast homolog Cdc53 (Lammer et al. 1998; Liakopoulos et al. 1998) have been shown to be modified by NEDD8, a ubiquitin homolog (Kamitani et al. 1997; Gong and Yeh 1999). When the *Cul-3* cDNA was cotransfected into h293 cells with HA-tagged NEDD8, the more slowly migrating of the two Cul-3 isoforms showed a decrease in its electrophoretic mobility that was consistent with its modification by the epitope-tagged NEDD8 as opposed to the endogenous (untagged) NEDD8. Cul-3 was then immunoprecipitated with anti-Cul-3 antibodies, and immunoblotted with anti-HA antibodies. We found that the more slowly migrating form of Cul-3 was specifically recognized by the anti-HA antibodies, and is therefore directly conjugated to NEDD8 (Fig. 2D).

Cul-3 binds to cyclin E in human cells

The interaction between Cul-3 and cyclin E was examined in mammalian cells. Expression vectors encoding full-length *Cul-3* and myc-epitope-tagged cyclins E, D1, A, or B were cotransfected into h293 cells (Fig. 3A). Cul-3 could be coimmunoprecipitated with cyclins D1 and E, but not cyclins A and B. The binding of Cul-3 to cyclin E was confirmed with multiple different antisera that recognized different parts of the cullin or cyclin, in reciprocal immuneprecipitations, and in experiments with epitope-tagged or untagged proteins (not shown). In these experiments, the cyclins were expressed in excess over their endogenous CDK partners, so the binding interactions we detected were between Cul-3 and free (unbound) cyclins. This was confirmed in parallel transfection experiments that showed that Cul-3 also bound to cyclin E R130A (not shown). In accord with the results of our two-hybrid screen, overexpression of Cdk2 together with wild-type cyclin E prevented the binding of Cul-3 to cyclin E (Fig. 3B). However, overexpression of Cdk2 did

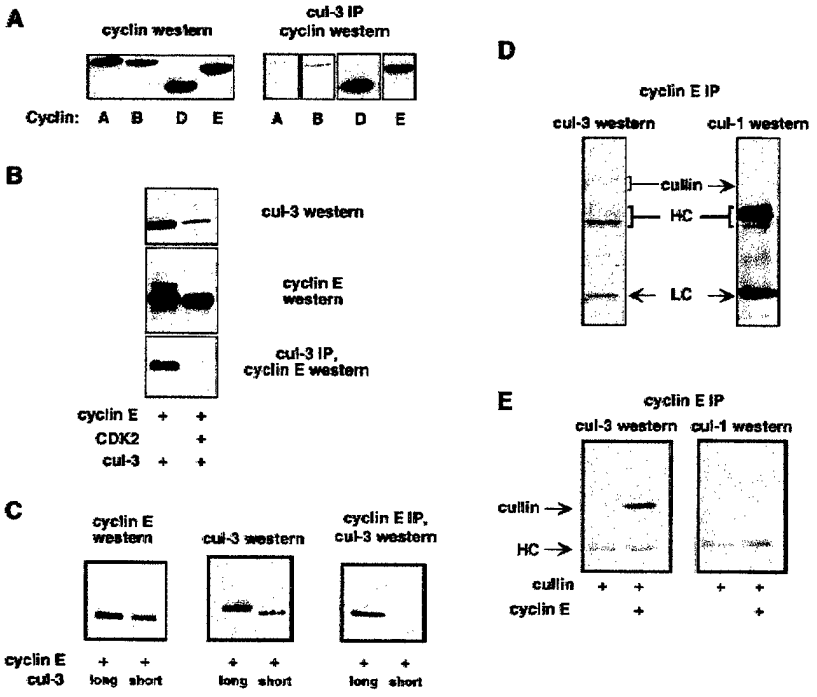

Figure 3. Cul-3 binds to cyclin E in mammalian cells. (*A*) Myc-epitope-tagged cyclins A, B, D, and E were cotransfected into cells with a Cul-3 cDNA. (*Left*) Western blot with the anti-myc tag antibody shows that the four cyclins were expressed at equivalent levels; (*right*) immunoprecipitates of Cul-3 contained cyclins E and D1, and not A or B. (*B*) Cotransfection of cells with cyclin E and Cdk2 prevented the binding of Cul-3 to cyclin E. (*C*) Cyclin E cDNA was cotransfected into h293 cells with either Cul-3 long cDNA or Cul-3 short cDNA. Only Cul-3 long binds to cyclin E. (*D*) A complex between Cul-3 and cyclin E can be detected in extracts from untransfected h293 cells. Binding of Cul-1 to cyclin E was not seen. (*E*) Epitope-tagged Cul-1 and Cul-3 were expressed in h293 cells together with cyclin E. Western blotting with an anti-epitope tag antibody showed that equivalent amounts of the two cullins were expressed (not shown). Immunoprecipitations showed that only Cul-3 bound to cyclin E. (HC) Position of immunoglobulin heavy chain.

not decrease binding of cyclin E R130A to Cul-3 (not shown). Thus, assembly of cyclin E into complexes with Cdk2 inhibits its interaction with Cul-3.

The abilities of the long and short alternatively spliced forms of Cul-3 to bind to cyclin E were tested by cotransfecting into h293 cells expression vectors encoding either form of Cul-3 together with myc-epitope-tagged cyclin E (Fig. 3C). Using anti-myc tag antibodies to specifically immunoprecipitate cyclin E, we found that the long form but not the short form of Cul-3 bound to cyclin E. This identified a region of Cul-3 in the amino terminus of the protein as being important for its interaction with cyclin E in mammalian cells. This region did not appear to be important for the interaction in yeast, because it was absent from our initial isolate of Cul-3 obtained in the two-hybrid screen. It is important to point out that in all tissues and cell lines that we have examined, the endogenous Cul-3 protein had an apparent molecular size consistent with that of the long form of Cul-3. Thus far we have not detected expression of the short form of Cul-3 protein. RT–PCR analyses confirmed that Cul-3 long was the major form of Cul-3 expressed in cells and tissues (not shown).

For technical reasons, it is often advantageous to study interactions between transfected, overexpressed proteins. Nevertheless, it is always important to confirm the relevance of those interactions by examining the state of the corresponding endogenous cellular proteins. To this end, extracts from untransfected h293 cells were prepared and endogenous cyclin E was immunoprecipitated. Immunoblotting the bound material with affinity-purified Cul-3 antibodies, demonstrated the presence of cyclin E-associated Cul-3 protein. (Fig. 3D). We do not yet know if this interaction is direct, or mediated by other proteins as has been seen in other cullin complexes (Feldman et al. 1997; Lyapina et al. 1989; Yu et al. 1998).

We also examined the ability of cyclin E to associate with Cul-1. This was of particular interest because Cul-1 is the closest homolog among the mammalian cullins to the yeast Cdc53 protein. Cdc53 has been shown to be involved in the ubiquitination of the yeast Cln G_1 cyclins, and therefore mammalian Cul-1 was considered to be a candidate for being involved in cyclin E ubiquitination in mammalian cells (Koepp et al. 1999). However, no binding of Cul-1 to cyclin E could be detected (Fig. 3D). The relative abilities of Cul-1 and Cul-3 to bind to cyclin E were also tested in a transfection assay. Expression vectors encoding *Cul-3* and *Cul-1* were cotransfected with myc-epitope-tagged cyclin E into h293 cells, and cyclin E immunoprecipitates tested for the presence of associated cullins (Fig. 3E). Just as we had seen with the endogenous proteins, transfected Cul-3 bound to cyclin E and Cul-1 did not. Therefore, Cul-1 does not seem to be involved in the pathway that recognizes free cyclin E.

Cul-3 stimulates ubiquitination of cyclin E

To study the effects of Cul-3 on the ubiquitination of cyclin E, expression vectors encoding Cul-3 and cyclin E were cotransfected into h293 cells. The presence of Cul-3 stimulated the accumulation of higher molecular weight forms of cyclin E, which for the following reasons are likely to be cyclin E-ubiquitin conjugates. First, these high molecular forms of cyclin E were similar to those that accumulated when the turnover of ubiquitin-conjugated proteins was prevented by pharmacological inhibition of the proteasome with MG-132 (Fig. 4A, left). Second, cyclin E and Cul-3 were cotransfected into h293 cells together with a plasmid expressing an HA-epitope-tagged form of ubiquitin. Cyclin E was immunoprecipitated with anti-cyclin E antibodies, and the recovered protein was immunoblotted with antibodies that recognize the HA epitope tag that was present on the cotransfected ubiquitin (Fig. 4A, right). This approach directly demonstrated that Cul-3 stimulated the accumulation of ubiquitin-conjugated cyclin E. When the same experiment was performed with cyclin A, Cul-3 had no effect on the accumulation of cyclin A-ubiquitin conjugates, although proteosomal inhibition with MG-132 readily caused accumulation of cyclin A-ubiquitin conjugates (Fig. 4B). Therefore, the effects of Cul-3 were specific for cyclin E, and overexpression of Cul-3 did not result in nonspecific inhibition of the proteasome.

Mutation of the CDK phosphorylation sites in cyclin E, including the critical threonine 380 residue, had no effect on either binding of Cul-3 to cyclin E (not shown) or on the ability of Cul-3 to stimulate cyclin E ubiquiti-

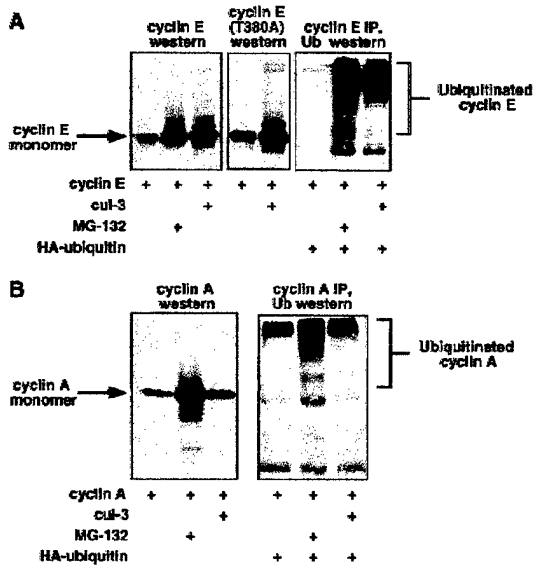

Figure 4. Cul-3 stimulates ubiquitination of cyclin E. (*A*) Cyclin E and Cul-3 cDNAs were cotransfected into h293 cells. (*Left*) Higher molecular weight forms of cyclin E are observed either in the presence of the proteasome inhibitor MG-132, or in the presence of Cul-3; (*right*) the same experiment was performed in the presence of HA-tagged ubiquitin. Both Cul-3 and MG-132 stimulate the conjugation of ubiquitin to cyclin E (*B*) Same as part *A* except that cyclin A was used instead of cyclin E. Cul-3 had no effect on the ubiquitination of cyclin A.

nation (Fig. 4A, middle). Therefore, the Cul-3 pathway for ubiquitination of free cyclin E is independent of cyclin E phosphorylation.

Construction of a Cul-3 knockout mouse

The *Cul-3* gene was disrupted by electroporation of murine embryonic stem cells (ES cells) with a targeting vector, pJS1052, in which amino acids 127–293 of the *Cul-3* gene were deleted and replaced with the *neomycin* gene under the control of the PGK promoter (see Materials and Methods for details). The targeting construct contained 6.8 kb of *Cul-3* genomic DNA as the upstream arm, and 1.4 kb of *Cul-3* genomic DNA as the downstream arm flanking the *neomycin* gene. Upstream of the long arm was the PGK promoter driving the *thymidine kinase* gene, which was used for counterselection to increase recovery of homologous integration events. Among the first 20 neomycin resistant ES cell colonies, 5 were shown by polymerase chain reaction to have undergone homologous recombination between pJS1052 and the chromosomal *Cul-3* gene. This was confirmed by Southern blot hybridization.

These five ES cell clones were microinjected into C57/BL blastocysts. Chimeric males were backcrossed to wild-type C57/BL females and two independent ES cell clones that successfully contributed to the germ line were used for subsequent experiments. The effects of the internal *Cul-3* deletion on *Cul-3* gene expression were assessed in embryonic fibroblasts prepared from E16 heterozygous mice. We used antibodies that had been specifically raised against amino acids 1–286 (which includes the part remaining in the Cul-3 knockout), and antibodies raised against the carboxy-terminal end of Cul-3 (see Materials and Methods) to assay for expression of Cul-3 protein by immunoblotting of whole cell extracts. Both antibodies detected full-length Cul-3 protein in the heterozygous MEFs, but neither detected any expression of a truncated form of Cul-3 protein that might have arisen from the mutated allele (not shown). We concluded that the deletion of amino acids 127–296 results in a null allele.

F_1 heterozygous mice containing one intact allele of *Cul-3* were intercrossed to obtain F_2 generation mice lacking Cul-3 protein. Among the first 100 progeny, no viable *Cul-3*$^{-/-}$ mice were obtained, whereas *Cul-3*$^{+/+}$ and *Cul-3*$^{+/-}$ animals were obtained at the expected frequencies. We concluded that homozygous deletion of the *Cul-3* gene caused an embryonic lethal phenotype.

Characterization of Cul-3$^{-/-}$ *embryos*

To determine the effects of the *Cul-3* deletion on development, we analyzed embryos obtained from pregnant females at various days of gestation following mating of F_1 heterozygous animals. Homozygous *Cul-3*$^{-/-}$ embryos were identified either by PCR of DNA prepared from yolk sacs or, for embryos at E7.5 and younger, by immunostaining with anti-Cul-3 antibodies (Fig. 5A).

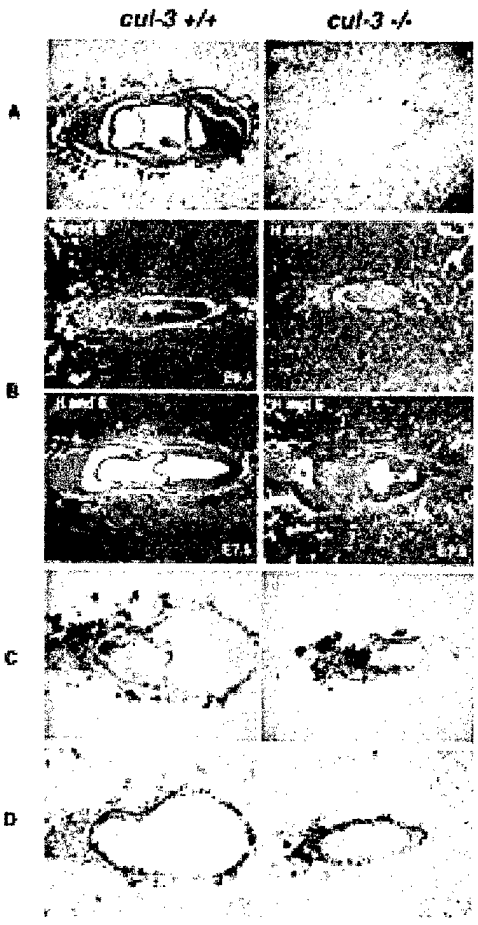

Figure 5. Embryonic lethal phenotype of *Cul-3*$^{-/-}$ mice. (*A*) Affinity-purified anti-Cul-3 antibodies were used to stain sections from E7.5 embryos. (*Left*) Cul-3 staining in a wild-type embryo. Note that the most intense nuclear staining is present in the extraembryonic ectoderm and the trophoblast giant cells. (*Right*) The *Cul-3*$^{-/-}$ embryo does not stain with Cul-3 antibodies. (*B*) H and E staining of embryos at either E6.5 (*top*) or E7.5 (*bottom*). (*C*) RNA in situ hybridization to detect expression of the H19 gene. H19-positive cells represent the extraembryonic ectoderm and trophectoderm lineages. (*D*) Detection of the trophectoderm lineage with Troma-1 antibodies.

Note that we were unable to distinguish between *Cul-3*$^{+/+}$ and *Cul-3*$^{+/-}$ embryos at stage E7.5 or younger, because Cul-3 immunostaining does not differentiate between these two genotypes. However, no systematic abnormalities were observed among the embryos that stained positively for Cul-3 protein and *Cul-3* heterozygous mice were represented in the expected proportion among the viable progeny of *Cul-3*$^{+/-}$ intercrosses, suggesting that *Cul-3*$^{+/-}$ embryos developed normally.

Cul-3$^{-/-}$ embryos were found at the expected Mendelian ratio until E7.5, after which *Cul-3*$^{-/-}$ yolk sacs contained partially degenerating or fully resorbed embryos. To further characterize the phenotype of Cul-3 mutant mice, we performed histological analyses of serial sec-

tions prepared from E6.5 and E7.5 embryos. E6.5 *Cul-3*$^{-/-}$ embryos were substantially smaller than wild-type embryos, and displayed markedly abnormal patterning both in the embryonic and extraembryonic tissues (Fig. 5B). E7.5 embryos were characterized in greater detail. *Cul-3*$^{-/-}$ embryos showed complete disorganization of the extraembryonic tissues, which were identified by RNA in situ hybridization with a probe for the H19 gene (Poirier et al. 1991; Fig. 5C), including the absence of an amnion and absence of the extraembryonic cavities. The trophectoderm, identified by staining with Troma-1 antibodies (Brulet et al. 1980), was present, but abnormally developed and trophoblast giant cells were more sparsely represented than in wild-type embryos (Fig. 5D). Gastrulation was also abnormal without clear evidence for the presence of embryonic mesoderm and endoderm.

Increased abundance of cyclin E protein in Cul-3$^{-/-}$ *embryos*

Serial sections of E7.5 embryos were stained with affinity-purified antibodies against cyclin E (Fig. 6—note that these images are not the same scale). In embryos expressing wild-type Cul-3 protein, cyclin E was most abundantly expressed in the trophoblast giant cells, and occasional, more weakly staining cells were detected scattered throughout the embryonic and extraembryonic tissues. *Cul-3*$^{-/-}$ embryos also expressed abundant cyclin E protein in the trophoblast cells, but equally high amounts were detected in the majority of cells in the ectoplacental cone and the extraembryonic ectoderm. In situ hybridization with cyclin E antisense RNA as probe did not reveal increased expression of cyclin E mRNA in *Cul-3*$^{-/-}$ embryos (not shown). Therefore, post-transcriptional events were the cause of elevated cyclin E protein expression in the *Cul-3*$^{-/-}$ embryos. Immunostaining did not detect increased expression of either cyclin A or cyclin D1 protein in the *Cul-3* mutant embryos (not shown).

Abnormal regulation of S phase in Cul-3$^{-/-}$ *embryos*

We studied the effects of the Cul-3 mutation on patterns of DNA replication in E7.5 embryos. Pregnant mice were injected intraperitoneally with BrdU. Four hours later embryos were removed, and sections stained with anti-BrdU antibodies to identify S-phase cells. A dramatic increase in the number of cells synthesizing DNA was detected in the extraembryonic ectoderm and ectoplacental cone of the *Cul-3*$^{-/-}$ embryos, the same cell types that expressed increased amounts of cyclin E protein (Fig. 6C).

Trophoblastic cells also expressed high amounts of cyclin E. Unlike the cells in the extraembryonic ectoderm, these cells undergo endoreduplicative cell cycles, increasing their DNA content many fold (MacAuley et al. 1998). DNA synthesis in these cells was studied in vitro, in cultured living blastocysts. Blastocysts were isolated 3.5 d.p.c., cultured on cover slips for 4 days, then fixed and immunostained for the expression of various proteins (Fig. 7). Also, 2 hr prior to fixing and staining, BrdU was added to the culture medium to label S-phase nuclei. Wild-type blastocysts developed both an inner cell mass, and migratory trophoblast giant cells (Fig. 7A); the latter were readily identifiable by their large nuclei, which result from genome endoreduplication, and also by their positive cytoplasmic immunostaining with Troma-1 antibodies (Fig. 7B). The trophoblast giant cells stained heterogeneously for cyclin E protein, consistent with the fact that cyclin E is known to oscillate in abundance during cycles of endoreduplication. BrdU staining revealed a good correspondence between the cyclin E positive cells and the cells that were in S phase (Fig. 7B).

Cul-3$^{-/-}$ blastocysts were identified either by PCR or by immunostaining for expression of the Cul-3 protein. These mutant blastocysts also developed an inner cell mass and migratory trophoblast cells. But, unlike the trophoblasts that expressed Cul-3 protein, the *Cul-3*$^{-/-}$ trophoblasts had much smaller nuclei, often the size of a normal diploid cell (Fig. 7A). Troma-1 immunostaining confirmed that these cells with small nuclei were trophoblasts (Fig. 7C). Every one of these small, *Cul-3*$^{-/-}$ trophoblast cells exhibited strong nuclear cyclin E immunostaining, suggesting that the normal cell cycle-dependent change in cyclin E levels was attenuated in these cells. Often the cyclin E staining was cytoplasmic as well as nuclear, and was more intense than in wild-type cells. Remarkably, none of the *Cul-3*$^{-/-}$ trophoblasts were positive for BrdU despite the high levels of cyclin E protein. Thus, their small nuclear size correlated with an absence of ongoing genome duplication. The disparate effects of cyclin E overexpression on S phase in mitotic versus endoreduplicative cell cycles is consistent with what has been observed previously in other model systems (Ohtsubo and Roberts 1993; Follette et al. 1998; Weiss et al. 1998).

Discussion

Ubiquitination of cyclin E by Cul-3

Both molecular and genetic approaches show that Cul-3 is important for the ubiquitination and degradation of mammalian cyclin E. Knockout of the *Cul-3* gene in mice caused an early embryonic lethal phenotype that was associated with increased amounts of cyclin E protein in the extraembryonic ectoderm and in the trophectoderm. The effect of the Cul-3 deletion was specific to cyclin E, as the expression of neither cyclin A nor cyclin D1 were increased.

The increased expression of cyclin E in cells lacking Cul-3 protein probably reflected a direct effect of Cul-3 on cyclin E turnover. We found that Cul-3 was tightly bound to cyclin E in vivo, and that increased expression of Cul-3 increased cyclin E ubiquitination. In contrast, Cul-3 neither bound to cyclin A nor had any effect on its ubiquitination. These observations support the idea that Cul-3 is part of an E3 protein–ubiquitin ligase that selects certain proteins, including cyclin E, for ubiquitination.

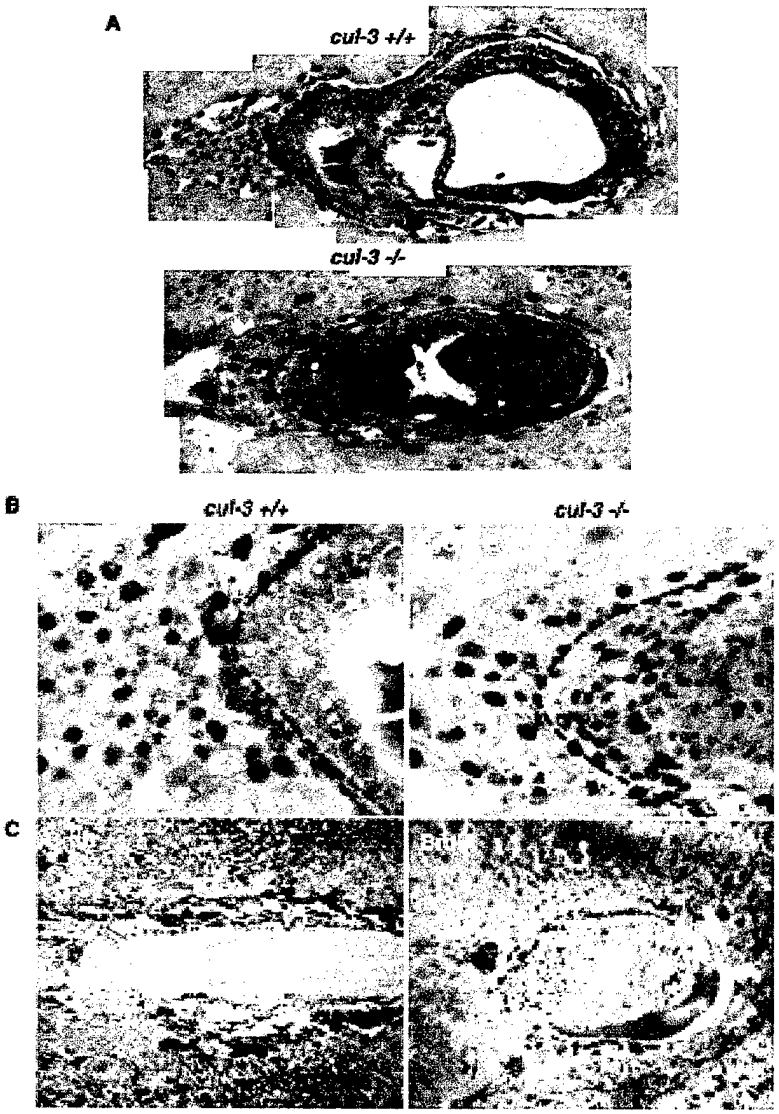

Figure 6. Expression of cyclin E protein in wild-type and *Cul-3*$^{-/-}$ *embryos. (A)* Montage images of either wild-type (*top*) or *Cul-3*$^{-/-}$ embryos (*bottom*) stained with anti-cyclin E antibodies. The images are not to scale. A broken line has been placed on both photos to delineate the border between the extraembryonic tissue and the trophectoderm as determined by staining with Troma-1 antibodies. Increased cyclin E protein is detected specifically in the extraembryonic ectoderm. (*B*) A high-magnification picture of the same embryos, to scale, to highlight the extraembryonic region in which cyclin E expression is increased in the *Cul-3*$^{-/-}$ embryo. (*C*) Wild-type (*left*) and mutant embryos (*right*) isolated from BrdU-injected animals were stained for BrdU incorporation. The *Cul-3*$^{-/-}$ embryo showed many more BrdU-positive cells, with the greatest number being in the same region shown to overexpress cyclin E.

We do not mean to imply that cyclin E is the only target of Cul-3, nor that cyclin E is selected for ubiquitination only by Cul-3. First, localization of Cul-3 to the Golgi apparatus suggests that Cul-3 may have additional substrates. These might include misfolded or improperly assembled proteins that arise during intracellular trafficking, proteins that are processed in preparation for export, or proteins that are modified by ubiquitin for retrograde transport (Hicke 1999). Second, overexpression of cyclin E is restricted to certain cell types in *Cul-3*$^{-/-}$ embryos. Other turnover pathways (perhaps involving other cullins) may be operative in those cells in which cyclin E levels are unaffected by the absence of Cul-3. This possibility is discussed further below.

More than one pathway for cyclin E ubiquitination?

Ubiquitination of cyclin E depends on two parameters; its binding to a CDK, and its state of phosphorylation on threonine 380 (Clurman et al. 1996; Won and Reed 1996). These two pathways may be governed by distinct ubiquitinating enzymes, one which recognizes a feature unique to unbound cyclin E, and the other which directly recognizes phosphorylated T380. We have shown previously that phosphorylation of T380 is required for ubiquitination of cyclin E bound to Cdk2, but not for ubiquitination of unbound cyclin E. Ubiquitination of a budding yeast G_1 cyclin, Cln2, occurs by a phosphorylation-triggered pathway in which an E3 protein–ubiquitin ligase, the SCF complex, binds directly to the phosphorylated cyclin (Deshaies et al. 1995; Willems et al. 1996; Skowyra et al. 1997, 1999). The fact that phosphorylation of T380 in cyclin E promotes the ubiquitination of cyclin E is consistent with the SCF paradigm, but direct evidence for the involvement of this pathway in the turnover of cyclin E is, thus far, lacking.

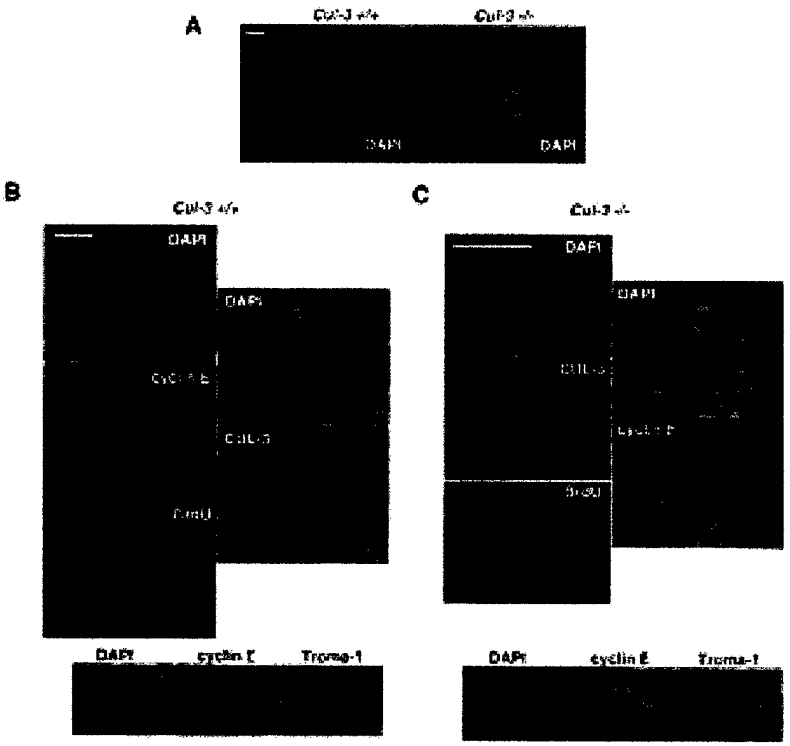

Figure 7. Analysis of wild-type and *Cul-3*$^{-/-}$ blastocysts. Bar, 100 μm. (*A*) DAPI staining of wild-type (*left*) and *Cul-3*$^{-/-}$ blastocysts. Images are to scale. Note the much smaller nuclei of the migratory *Cul-3*$^{-/-}$ trophoblasts. (*B*) Wild-type trophoblast cells are stained with DAPI, anti-cyclin E, and anti-BrdU antibodies (*left*). (*Right*) Blastocysts stained with DAPI and Cul-3 antibody to show that the giant cells normally express Cul-3. (*Bottom*) The giant cells in wild-type blastocysts also express Troma-1. (*C*) Same as *B*, except that *Cul-3*$^{-/-}$ blastocysts are shown. The mutant trophoblasts do not stain for Cul-3 or BrdU.

A second pathway, one which involves ubiquitination and degradation of proteins that are separated from their normal binding partners, may also be critically important. This pathway was initially recognized as being crucial for the rapid turnover of proteins within the endoplasmic reticulum that are either misfolded or incorrectly assembled into multiprotein complexes (Hurtley and Helenius 1989; Bonifacino and Weissman 1998). One example is the rapid destruction of T-cell receptor α chains that fail to assemble into complexes with other receptor subunits (Bonifacino et al. 1989, 1990). This general idea was then extended to include nuclear proteins including cyclin E, which is protected from ubiquitination when assembled with Cdk2 (Clurman et al. 1996), and E2F-1, which is protected from ubiquitination when bound to Rb (Hofmann et al. 1996). The α2 transcription factor in budding yeast is another example of a protein whose ubiquitination is controlled in this way (Johnson et al. 1998; Laney and Hochstrasser 1999). Within the α2 protein is the Deg1 sequence, which is a recognition motif for an E3 ubiquitin–protein ligase. When the α2 protein is monomeric, the Deg1 sequence is exposed, and α2 is rapidly ubiquitinated and turned over by the proteasome. However, when α2 binds to its partner, the a1 transcription factor, the Deg1 motif is buried in the heterodimer interface, and the protein is protected from ubiquitination. In the case of cyclin E, Cul-3 recognizes and stimulates the ubiquitination of unbound cyclin E, not cyclin E within cyclin E–Cdk2 complexes. Cyclin E, like α2, might contain an instability determinant that is masked in the cyclin E–Cdk2 complex. Alternatively, it is possible that other features of the cyclin E–Cdk2 complex, such as its kinase activity, might downregulate the interaction between cyclin E and Cul-3. It is important to emphasize that this pathway for cyclin E ubiquitination is unlikely to be limited to the destruction of unfolded or otherwise nonfunctional protein. Cells lacking Cul-3 appear to accumulate excess, biologically active cyclin E as evidenced by the misregulation of S phase in *Cul-3*$^{-/-}$ embryos. It therefore seems that the unbound cyclin E is at least potentially active, and it is crucial for the cell to limit the size of this pool.

Other features of Cul-3 also suggest that there may be significant differences between its mechanism of action and the phosphorylation-dependent pathway controlled by the SCF. Among the mammalian cullins, Cul-1, not Cul-3, is most closely related to Cdc53 (the cullin component of the budding yeast SCF). Cul-1, like Cdc53, binds to Skp1 and to the E2 ubiquitin-conjugating enzyme Cdc34 (Lisztwan et al. 1998; Michel and Xiong 1998; Yu et al. 1998). Moreover, human Cul-1 can complement mutations in budding yeast Cdc53 and Cul-1 is involved in the phosphorylation-triggered ubiquitination of proteins in mammalian cells, including E2F-1, B-catenin, and IkB (Hatakeyama et al. 1999; Kroll et al. 1999; Latres et al. 1999; Suzuki et al. 1999; Winston et al. 1999). Cul-3, on the other hand, does not bind to Skp-1 (Michel and Xiong 1998) or Cdc34 (our unpublished observations), does not complement mutations in Cdc53 (J. Singer et al., unpubl.), and does not require substrate phosphorylation for binding and ubiquitination. It therefore seems possible that these two pathways

for ubiquitinating protein substrates will be governed by very distinct E3 enzymes. In some embryonic tissues, like the embryonic ectoderm, loss of Cul-3 had no apparent effect on cyclin E abundance, suggesting that the relative importance of different pathways for controlling cyclin E abundance may vary among different cell types. Phosphorylation-triggered ubiquitination of cyclin E, perhaps mediated by Cul-1, may be a counterpart to the Cul-3 pathway that specifically recognizes unbound cyclin E. The above discussion is not intended to exclude the possibility that Cul-3 is also involved in the ubiquitination of phosphorylated cyclin E. We have suggested that phosphorylation may trigger the separation of cyclin E from its CDK partner, in which case the two ubiquitination pathways would converge.

S-phase regulation by Cul-3

Regulation of S phase is abnormal in *Cul-3*$^{-/-}$ embryos. In extraembryonic ectodermal cells, which undergo a standard mitotic cycle, the loss of Cul-3 results in a greatly elevated frequency of cells in S phase. In the trophoblast giant cells, which endoreduplicate their genomes, the loss of Cul-3 has the opposite effect of imposing a block to S-phase entry. These paradoxical results can both be explained by elevated expression of cyclin E.

Most current models of cell cycle regulation incorporate the idea that each round of DNA replication (i.e., each S phase) is regulated by two CDK-dependent steps. The first step requires that CDK activity be low or absent, creating an environment permissive for the assembly of initiation complexes at replication origins. The second step requires active CDKs to initiate DNA synthesis from those primed origins and to simultaneously establish an environment refractory to assembly of new initiation complexes. Rounds of genome duplication, as occur either during endocycles or in consecutive mitotic cycles, would then each require that CDK activity oscillate between a state permissive for assembly of initiation complexes and a state permissive for starting DNA synthesis.

This model predicts that constitutively high CDK activity would interrupt the replication cycle by not allowing assembly of new initiation complexes at replication origins. This was tested in the salivary gland cells of developing *Drosophila* embryos (Follette et al. 1989; Weiss et al. 1998). These cells endoreduplicate their genomes, each round of S phase being preceded by a fall in cyclin-E activity and then initiated in concert with a rise in cyclin E activity. Overexpression of cyclin E prevented this oscillation and the endoreduplication cycles were blocked. We suggest that S-phase entry in *Cul-3*$^{-/-}$ trophoblasts is similarly prevented by the constitutively elevated amounts of cyclin E in these cells.

In contrast to what happens in cells undergoing endoreduplication, increased expression of cyclin E in cells undergoing a typical mitotic cycle decreases the duration of G_1 and increases the percentage of cells in S phase (Ohtsubo and Roberts 1993; Resnitzky and Reed 1995). This result is consistent with what we observed in the *Cul-3*$^{-/-}$ cells of the extraembryonic ectoderm, which have increased cyclin E and extra S-phase cells. There are various ways to reconcile the seemingly contradictory effects of elevated cyclin E in mitotic cycles and endocycles. Most explanations focus on a key difference between these two types of cell cycles—the presence or absence of an M phase—and postulate that cyclin E somehow becomes functionally inactivated during mitosis, permitting the replication cycle to continue despite the constitutive presence of the cyclin. One interesting idea is that during mitosis, nuclear cyclin-CDK enzymes are dispersed into the cytoplasm by nuclear envelope breakdown, resulting in a de facto oscillation in CDK activity (Hua et al. 1997).

Materials and methods

Two-hybrid screen

The R130A cyclin E cDNA was cloned in frame to the *LexA* gene in the vector BTM116. Yeast cells containing LexA-binding sites in the HIS3 promoter and carrying a plasmid containing a LexA-dependent promoter driving *lacZ* expression were transformed with the cyclin E bait as well as a mouse embryonic cDNA library. Transformed cells were grown under selection for the plasmids overnight and plated for the two-hybrid interaction the next day. Potential candidates were screened for β-galactosidase activity and positive clones were rechecked with a LexA lamin bait for specificity.

Antibodies

hCul-3 was subcloned as three parts in frame with a polyhistidine tag in the vector pET16; the amino-terminal portion contains amino acids 1–286, the middle portion amino acids 287–553, and the carboxy-terminal portion amino acids 554–768. All three peptides were expressed in the bacterial strain BL21(DE3) and cell lysates were either passed over nickel columns, after which Cul-3 was eluted with imidazole, or the lysates were mixed with SDS sample buffer and separated from other cellular proteins by electrophoresis followed by electroelution of the Cul-3 protein. Purified protein was then used for inoculation into rabbits. To affinity purify antibodies, a strip of membrane containing the Cul-3 peptide was incubated with serum and the bound antibodies eluted with low pH glycine. Cells studied included human diploid fibroblasts, human diploid microvascular endothelial cells, human diploid umbilical vein endothelial cells, HeLa cells, h293 cells, U2-OS cells, NIH-3T3 cells, and primary mouse embryo fibroblasts (MEFs).

The following antibodies were used in these experiments: monoclonal anti-myc tag (9E10) and rabbit polyclonal anti-cyclin E (Clurman et al. 1996); monoclonal anti-cyclin E (M20) (Santa Cruz Biotechnology); monoclonal anti-Troma-1 (Developmental Studies Hybridoma Bank, University of Iowa); rabbit polyclonal anti-HA tag (HA.11) (Berkeley Antibody Company); rabbit polyclonal anti Cul-1 (J. Michel and Y. Xiong, University of North Carolina, Chapel Hill).

Transient transfections, cell lysis, Western blots, and immunoprecipitations were performed as described previously (Clurman et al. 1996). Immunoprecipitations were routinely checked for the presence of the immuneprecipitated protein, and all interactions between two transfected proteins were shown to be dependent on, or stimulated by, transfection of both proteins.

Immunofluorescence

Immunofluorescence was performed on cells adhering to coverslips by fixing in 4% paraformaldehyde in PBS for 10 min followed by treatment with 0.2% Triton X-100 for an additional 10 min. Cells were then blocked with 1% BSA and 20% goat serum for 30 min. Coverslips were inverted onto 20 µl of primary antibody and incubated for 1 hr. The coverslips were washed and placed on 20 µl of secondary antibody for an additional hour. They were then incubated with DAPI (4',6-diamidino-2-phenylindole) for 5 min, dehydrated in 100% MeOH, followed by mounting in 6 µl of Vectashield and sealed with nail polish. Blastocysts were fixed in 3.7% formaldehyde for 15 min, permeabilized in 0.3% Triton X-100, and stained as above. Images were obtained on a Nikon E800 fluorescent microscope with a digital camera (SenSys) and Metamorph software.

Immunohistochemistry

Embryos (7.5 day) were prepared by timed matings with *Cul-3* heterozygous animals. The pregnant uterus was surgically removed and the individual decidua were separated and fixed in 4% paraformaldehyde overnight at 4°C. Embryos were then embedded in paraffin blocks and cut into 4 µm sections. The sections were placed on slides to be used for either antibody staining or RNA in situ hybridization.

For antibody staining, the sections were deparaffinized and placed into a 3% solution of hydrogen peroxide in methanol for 10 min. Slides were immersed in 10 mM citrate buffer and boiled for 10 min in a microwave oven. The slides were allowed to cool and were then treated with 5% serum followed by an overnight incubation at 4°C with primary antibody. Sections were then incubated with biotinylated secondary for 1 hr and avidin–HRP complex for an additional 30 min. The slides where then immersed in DAB (3,3'-diaminobenzidine)/$NiCl_2$ solution for 3 min, rinsed, dehydrated, and mounted.

In situ hybridizations were performed by deparaffinizing the sections and treating them with proteinase K for 5 min. The sections were then fixed in 4% paraformaldehyde for 15 min. The slides were then placed in prehybridization solution for 2 hr at 65°C in a sealed humidified container. The sections were then hybridized overnight with digoxigenin-labeled riboprobe. The slides were washed, blocked, and incubated overnight with alkaline phosphatase conjugated anti-digoxigenin antibody. The RNA was visualized by staining with a NBT/BCIP solution for 8 hr followed by dehydration and mounting.

For the H19 in situs a 2-kb *Eco*RI fragment from the carboxyl terminus of the cDNA was used for making the riboprobe. The cyclin E riboprobe was made from the last 600 bp of the mouse cyclin E cDNA. The antibodies used for cyclin A (c19), cyclin D1 (72–13G), and cyclin E (M20) immunohistochemistry were from Santa Cruz Biotechnology, or cyclin E antibody described previously was used (Clurman et al. 1996).

Targeted mouse gene disruption

A 22-kb *Not*I fragment containing a portion of the *Cul-3* gene was obtained from a mouse 129/Sv λ genomic library with portions of the *Cul-3* cDNA as a probe. Sequencing of the genomic insert was done partially by shotgun cloning *Hae*III–*Alu*I partial digest fragments of the 22-kb *Not*I fragment into the *Eco*RV site of pBSII (Stratagene) and sequencing 100 individual clones with both the T7 and T3 sequencing primers. The sequencing data was then assembled into multiple contigs with Sequencher software. Alignments with the *Cul-3* cDNA sequence and sequence analysis was performed with DNA Strider. pJS1052 was constructed by cloning a 6.8-kb *Eco*RI fragment as the upstream arm that ended at amino acid number 126 of the *Cul-3* coding region and a downstream 1.4-kb *Xba*I–*Eco*RV *Cul-3* genomic fragment that contained coding regions starting with amino acid number 294 of the Cul-3 protein into the targeting vector pPNT. The vector was linearized with *Not*I and transfected into XY AK7 ES cells with electroporation. The ES cells were then selected in 400 µg/ml G418 and 0.2 µM FIAU. ES cell colonies with homologous recombination were identified by PCR amplification of a 2-kb fragment with a primer from the *neomycin* gene (pgk2, CCCTTCCCAGCCTCTGAG) and a primer from *Cul-3* genomic DNA (cul3PCR2, CAACTCATACATTCACACATGG). PCR reactions were performed for 40 cycles (93°C for 30 sec; 57°C for 30 sec; 65°C for 2 min). ES cells were introduced into 5 d.p.c. C57/B6J mouse embryos. Germ-line transmission, as determined by PCR, was identified in chimeric males obtained from two independent clones that were used for subsequent experiments.

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Proteasomal Turnover of p21^{Cip1} Does Not Require p21^{Cip1} Ubiquitination

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Summary

The Cdk inhibitor p21^{Cip1} is an unstable protein. Pharmacologic inhibition of the proteasome increases the half-life of p21 from less than 30 min to more than 2 hr and results in the accumulation of p21–ubiquitin conjugates. To determine whether ubiquitination was required for proteasomal degradation of p21, we constructed mutant versions of p21 that were not ubiquitinated in vivo. Remarkably, these mutants remained unstable and increased in abundance upon proteasome inhibition, indicating that direct ubiquitination of p21 is not necessary for its turnover by the proteasome. The frequently observed correlation between protein ubiquitination and proteasomal degradation is insufficient to conclude that ubiquitination is a prerequisite for degradation.

Introduction

The periodic expression of cell cycle proteins is a central feature of cell cycle control. Targeted proteolysis by the ubiquitin–proteasome system has emerged as a key determinant of this periodicity (King et al., 1996a; Peters, 1998; Koepp et al., 1999). Ubiquitin conjugation regulates diverse biological systems and occurs in a series of highly regulated enzymatic reactions, thereby allowing precise substrate targeting in appropriate physiologic contexts (Hochstrasser, 1996; Hershko and Ciechanover, 1998). Several criteria are used to determine if a specific protein is degraded by the ubiquitin–proteasome system: the decrease in the rate of substrate turnover in cells caused by inhibition of the system with chemical inhibitors or by genetic mutations; detection of ubiquitin-substrate conjugates in cells, usually following proteasome inhibition; reconstitution of ubiquitination and/or proteasomal degradation of substrates in cell extracts in vitro; and association of putative substrates with ubiquitinating enzymes.

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Many mammalian (e.g., cyclins [E,D,A,B] p27, p21, E2F, Rb, and p53) and yeast (e.g., Clns, Clbs, Sic1, and Far1) cell cycle proteins are ubiquitinated, and their degradation is dependent upon the proteasome in vivo (King et al., 1996a; Peters, 1998; Koepp et al., 1999; and references therein). In some cases (B-type cyclins, p27, Sic1, Far1, and Cln2), ubiquitination and/or proteasomal degradation have also been demonstrated in extracts that recapitulate features of cellular regulation. Furthermore, in yeast cells the degradation of proteins such as Far1 and Sic1 requires specific ubiquitination enzymes, suggesting that turnover of these proteins is directly regulated by ubiquitination. Nevertheless, it is difficult to demonstrate that direct ubiquitination of a given protein is a prerequisite for its degradation in vivo, and in most cases, particularly for mammalian proteins, this has not been shown.

Results

p21 Is an Unstable Protein that Exhibits Proteasomal Turnover and Is Ubiquitinated In Vivo

p21 belongs to the Cip/Kip family of cyclin-dependent kinase (Cdk) inhibitors and mediates cell cycle arrest in response to stimuli such as activation of the p53 tumor suppressor (Sherr and Roberts, 1995). p21 meets at least two of the criteria for ubiquitin–proteasome regulation in that proteasome inhibition results in both p21 accumulation and appearance of p21–ubiqutin conjugates in vivo (Blagosklonny et al., 1996; Maki and Howley, 1997; Cayrol and Ducommun, 1998; Rousseau et al., 1999). Treatment of human diploid fibroblasts with two chemically distinct proteasome inhibitors (the peptide aldehyde MG-132 or the more specific proteasome inhibitor clasto-lactacystin-β-lactone) resulted in the accumulation of endogenous p21 protein (Figure 1A). This was due solely to increased p21 stability. Its half-life increased from less than 30 min to greater than 2 hr (Figure 1B) whereas its rate of synthesis remained unchanged (Figure 1A). In contrast, a radiomimetic dose of actinomycin D, which induces p53, increased the rate of p21 synthesis, but not its half-life (Figure 1A).

Although very low abundance p21–ubiquitin intermediates can be detected in some cell types, we did not detect ubiquitinated endogenous p21 in human diploid fibroblasts after proteasome inhibition, even though p21 was dramatically stabilized. It can be difficult to detect ubiquitin–protein conjugates in vivo due to rapid removal of the ubiquitin chains by deubiquitinating enzymes. We therefore utilized exogenously expressed p21 to assess the role of ubiquitination and specific amino acid residues in p21 turnover. The half-life of transfected p21 was also less than 30 min, and its abundance increased by proteasome inhibition (Figures 1C and 1D). In this case, however, p21–ubiquitin conjugates were readily detected (Figure 1D). These high-molecular weight species contained both p21 and ubiquitin as shown by their altered molecular weight when an HA epitope-tagged ubiquitin molecule was coexpressed with p21 (Figure 1D).

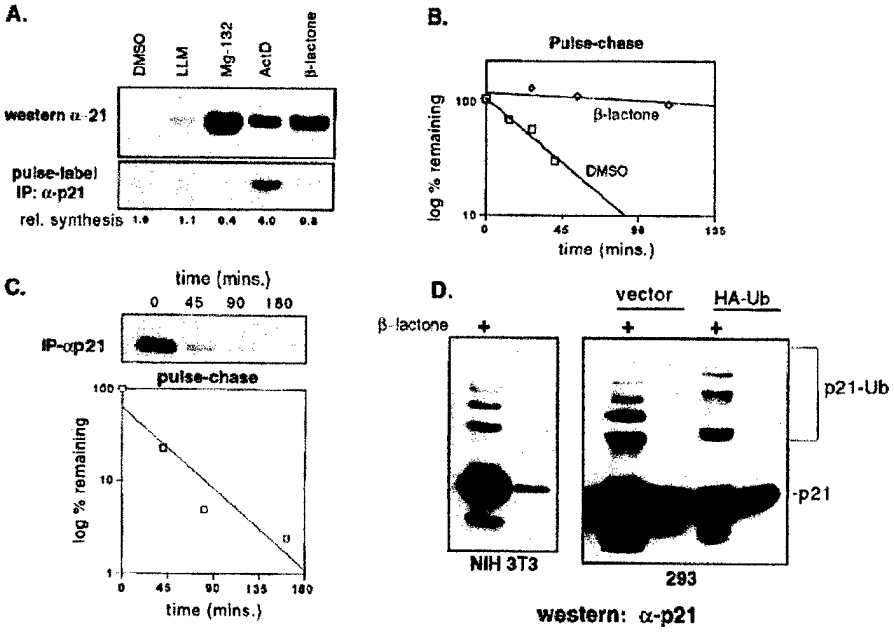

Figure 1. Proteasomal Turnover and Ubiquitination of p21

(A) Human diploid fibroblasts were treated with the indicated drugs for 16 hr (DMSO, LLM, Mg-132, and actinomycin D) or 5 hr (β-lactone) and then pulse labeled for 10 min. Top panel, Western analysis of p21 abundance; bottom panel, amount of p21 protein synthesized during pulse.

(B) Pulse–chase analysis of endogenous p21 in human diploid fibroblasts ± β-lactone.

(C) Pulse–chase analysis of transfected p21 protein in 293 cells.

(D) Transfected p21 is proteasome dependent and ubiquitinated. Left, NIH 3T3 cells were transfected with CS2p21 and treated with β-lactone or solvent control. Right, 293 cells were cotransfected with p21 expression vector and either HA-ubiquitin or control vector, and treated with β-lactone as shown. Ubiquitinated p21 species are shown (p21-Ub).

p21 Protein that Cannot Be Ubiquitinated Remains Unstable

If p21 ubiquitination is a prerequisite for its proteasomal turnover, then inhibition of the ubiquitination pathway should stabilize p21. We tested this hypothesis using four different approaches, all of which suggested that proteasomal degradation of p21 did not require p21 ubiquitination. First, we utilized a ubiquitin mutant (UbR7) lacking lysines that inhibits multiubiquitination of cyclin B, but not monoubiquitination (T. McGarry, personal communication). As a control, cotransfection of this mutant ubiquitin increased the steady-state abundance and half-life of cyclin E, a substrate of the cullin family of E3 ubiquitin ligases (Clurman et al., 1996; Won and Reed, 1996; Dealy et al., 1999; Singer et al., 1999; Wang et al., 1999) (Figure 2A). In contrast, overexpression of UbR7 did not increase the abundance of p21. These data suggest that direct multiubiquitination is required for cyclin E degradation, but not for p21, although we cannot rule out the possibility that cyclin E and p21 are differentially sensitive to conjugation with UbR7.

Second, we mutated all six lysines in p21 (the sites of potential ubiquitin conjugation) to arginine (p21K6R). p21K6R bound, inhibited, and was phosphorylated by cyclin–Cdk complexes like wild-type p21, indicating that its structure and activity were not severely affected by these six conservative substitutions (Figure 2B). No p21–ubiquitin conjugates of p21K6R were detected in vivo after proteasome inhibition in either NIH3T3 or 293 cells, even when highly overexpressed and when the film was overexposed (Figure 2C and data not shown), indicating that ubiquitination of p21 was prevented by mutation of the lysines. Surprisingly, p21K6R not only remained unstable, but its abundance also increased in response to proteasome inhibition indistinguishably from wild-type p21 (Figure 2C). Furthermore, the half-life of both wild-type p21 and p21K6R increased from less than 30 min to greater than 2 hr after proteasome inhibition (Figure 2D).

In parallel, we also examined the degradation of a lysineless mutant of cyclin E (cyclin EΔK). Mutating all of the lysines in cyclin E prevented formation of cyclin E–ubiquitin conjugates (Figure 2E). However, unlike p21, the abundance of cyclin EΔK was no longer responsive to proteasome inhibition, consistent with a major role for ubiquitination in cyclin E turnover.

Third, we examined the role of p21 ubiquitination under more physiologic conditions utilizing stable retrovirally transduced cell lines. The retroviral p21 was of human origin and introduced into mouse cells, allowing us to specifically monitor expression of the exogenous and endogenous p21 proteins by using species-specific antibodies and the different electrophoretic mobilities

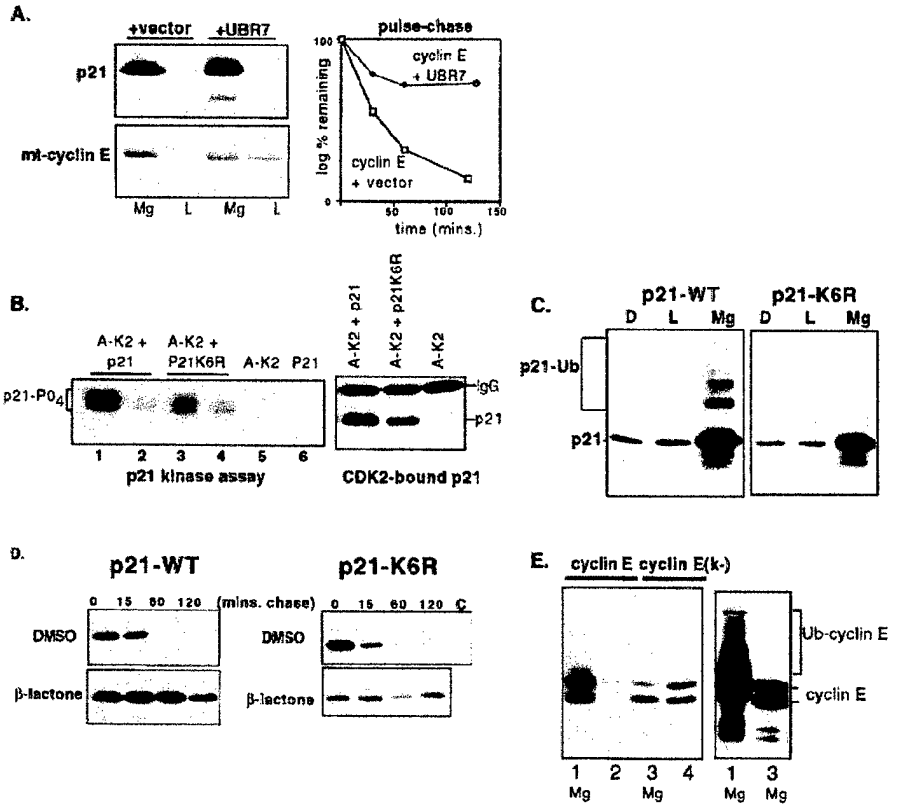

Figure 2. Inhibition of p21 Ubiquitination Does Not Affect Its Turnover

(A) 293 cells were cotransfected with either p21 or cyclin E and either empty vector or lysineless ubiquitin (UBR7). Cells were treated with either the control peptide aldehyde LLM (L) or MG-132 (Mg). Pulse–chase: half-life of transfected cyclin E ± UBR7 in 293 cells. To minimize possible effects of UBR7 on cyclin E turnover other than ubiquitination, cyclin E(R130A) was used because the turnover of this protein is not regulated by Cdk2 binding or phosphorylation (Clurman et al., 1996).

(B) p21K6R binds and inhibits cyclin A-Cdk2. 293 cells were transfected with 0.5 μg (lanes 1 and 3) or 2 μg (lanes 2 and 4) of p21 or p21K6R, 5 μg of CS2myc-cyclin A, and 3 μg of CMV-Cdk2 as indicated. Lysates were immunoprecipitated with anti-myc tag and than subjected to either a p21 kinase assay (left) or anti-p21 Western blot (right).

(C) Lysineless p21 is not ubiquitinated but remains proteasome dependent. NIH3T3 cells were transfected with either p21 or p21K6R and treated with DMSO (D), LLM (L), or Mg-132 (Mg).

(D) The half-lives of p21K6R and wt-p21 are similar. 293 cells were transfected with either CS2p21 or CS2p21K6R and treated with β-lactone as indicated.

(E) Lysineless cyclin E is stable and does not form ubiquitin conjugates. Left, 293 cells were transfected with vectors expressing either cyclin E or cyclin EΔK. Mg-132 treatment is indicated. Right, overexposure of lanes 1 and 3.

of human and mouse p21 (see Experimental Procedures). We directly compared the abundance of the exogenous wt-p21 to endogenous mouse p21 in the same cell population and found that the exogenous p21 was expressed at levels equal to or less than endogenous p21 (Figure 3A). We next observed that the exogenous wt-p21 and p21K6R were expressed at similar levels in the respective cell pools and that the infected cells proliferated normally, consistent with the observed levels of expression (Figures 3A–3C and data not shown). Furthermore, the retroviral wt-p21 and p21K6R bound to similar amounts of endogenous Cdk2, indicating that both proteins were incorporated into Cdk–complexes equivalently (Figure 3B).

We then measured turnover of the retrovirally expressed p21 and p21K6R in these stable cell lines. Treatment of cell pools expressing either wt-p21 or p21K6R with Mg-132 or β-lactone increased the steady-state accumulation of both proteins as assayed by Western blotting and by indirect immunofluoresence (Figures 3C and 3E and data not shown). Additionally, the half-lives of p21K6R and endogenous mouse p21 were almost indistinguishable within the same cells (Figure 3D). Thus, the turnover of p21K6R mirrored that of wild-type p21 under near-physiologic expression conditions.

Fourth, in rare cases, ubiquitin can be conjugated to proteins via the N-terminal α-NH2 group, although most mammalian proteins are N^{α} acetylated, and their N termini cannot be ubiquitinated (Brown, 1979; Hershko et al., 1984; Tsunasawa and Sakiyama, 1984). The N terminus was recently identified as the key ubiquitination site for the MyoD protein (Breitschopf et al., 1998). An important distinction between MyoD and p21 is that lysineless MyoD is ubiquitinated in vivo, whereas

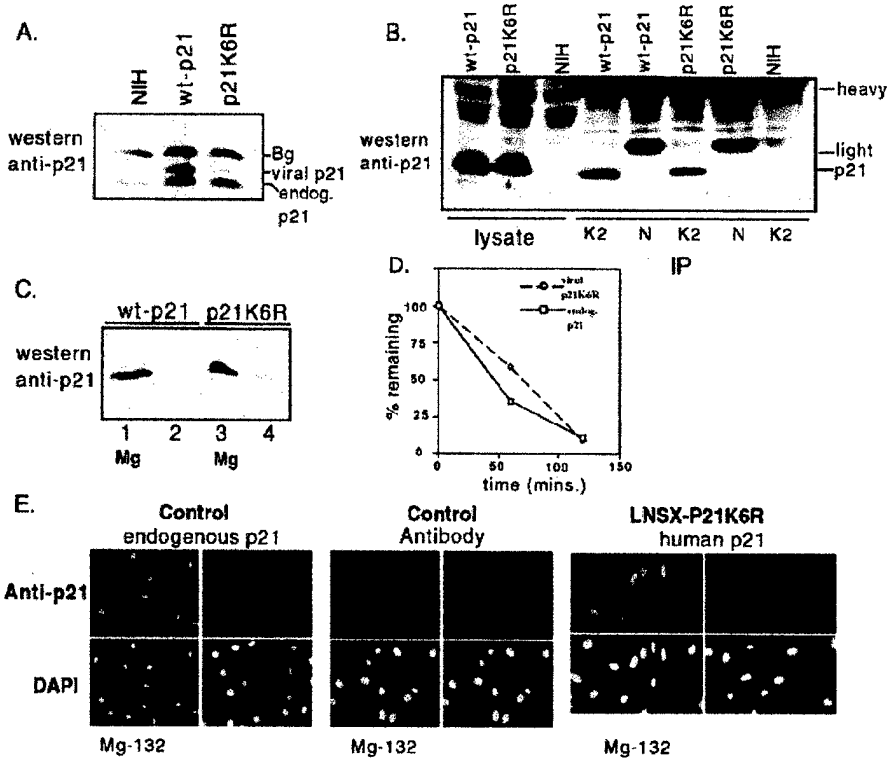

Figure 3. p21K6R Remains Proteasome Sensitive at Physiologic Levels of Expression

(A) NIH 3T3 cells transduced with LNSX-p21 and LNSX-p21K6R and control NIH3T3 cells were analyzed with polyclonal anti-p21(C-19), which recognizes exogenous p21 (human origin) and endogenous murine p21. Bg, background band. This antibody detects p21K6R poorly, but monoclonal anti-p21 (Transduction Labs) detects similar amounts of p21 and p21K6R in (B) and (C).

(B) Retrovirally expressed p21 and p21K6R are incorporated into Cdk-containing complexes. Cell lysates were immunoprecipitated with nonimmune serum (N) or anti-Cdk2 antibody (K2), followed by Western analysis with monoclonal anti-p21 (Transduction Labs).

(C) Retrovirally expressed wt-p21 and p21K6R are proteasome sensitive. The indicated cells were treated with Mg-132, followed by Western analysis with monoclonal anti-p21 (Transduction Labs).

(D) The half-lives of retroviral p21K6R and endogenous p21 are similar.

(E) Control NIH3T3 cells were stained with polyclonal anti-p21(C-19) (left panel), or monoclonal anti-p21 (Transduction Labs) (middle panel). LNSX-p21K6R cells were stained with monoclonal anti-21 (Transduction Labs) (right panel). Mg-132 treatment is indicated. The endogenous mouse p21 is detected by polyclonal but not monoclonal anti-p21 and is sensitive to proteasome inhibition. The exogenous human p21K6R is detected by monoclonal anti-p21 and is similarly responsive to proteasome inhibition.

p21K6R is not. Additional evidence that N-terminal ubiquitination is not important for p21 turnover was obtained by N-terminal epitope tagging p21 and p21K6R. This manipulation was previously shown to stabilize MyoD in vivo by preventing its amino-terminal ubiquitination (Breitschopf et al., 1998). However, the abundance and half-life of the N-terminal p21 fusion proteins remained sensitive to proteasome inhibition (Figures 4A and 4B). Furthermore, the N termini of human p21, murine p21, myc-p21 and HA-p21 (Met-Ser, Met-Ser, Met-Gly, and Met-Ala, respectively) all contain residues most frequently found in N-acetylated proteins (Tsunasawa and Sakiyama, 1984; Bradshaw et al., 1998). Therefore, N-terminal ubiquitin conjugation is unlikely to be the mechanism of p21 turnover since deletion of the p21 lysines eliminates p21–ubiquitin conjugate formation, indicating that ubiquitin is not attached to another site on the protein, and since the abundance and stability of N-terminal p21 fusion proteins remained sensitive to proteasome inhibition.

Regulation of p21 Turnover

We have begun to address the mechanisms that might direct p21 to the proteasome, if it is not via direct ubiquitination. These experiments have revealed that proteasomal turnover of p21 depends upon its localization to the nucleus. A p21 C-terminal truncation mutant lacking its nuclear localization signal, p21 (1–141), differed from wild-type p21 in two respects; it accumulated almost exclusively in the cytoplasm (Figure 4C), and its abundance was largely insensitive to proteasome inhibition (Figure 4D). To address the possibility that deletion of the C-terminal 25 amino acids removed an "instability" sequence, we added an SV40 NLS to p21(1–141) to redirect it to the nucleus. The readdition of an NLS effectively

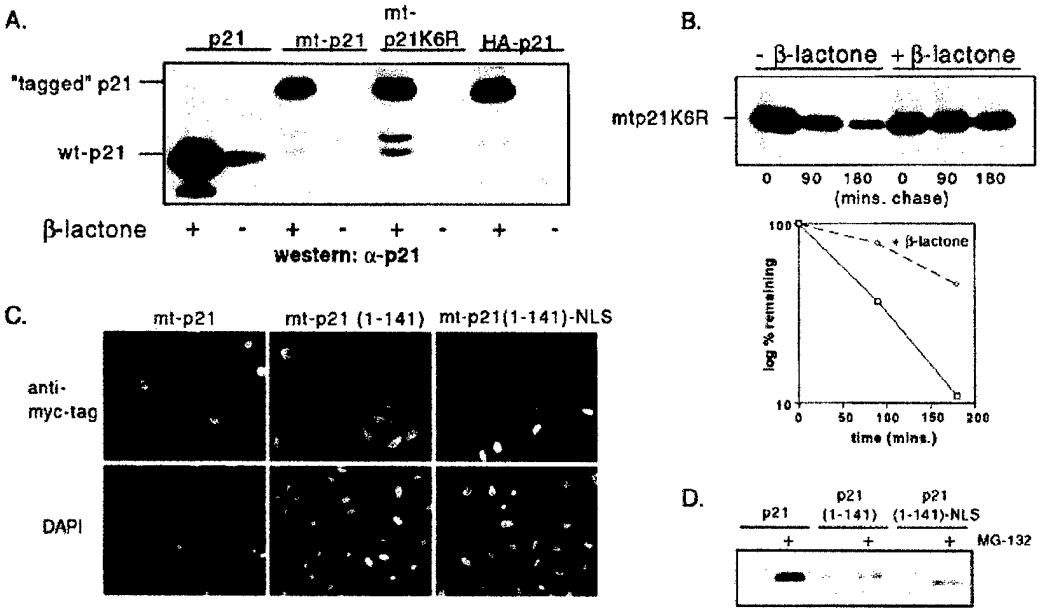

Figure 4. Proteasomal Regulation of N-Terminal p21 Epitope Tag Fusions

(A) 293 cells were transfected with expression vectors for the indicated epitope-tagged proteins and treated with β-lactone as shown.

(B) Pulse-chase analysis of mt-p21K6R ± β-lactone.

(C) A C-terminal deletion of p21 renders the protein cytoplasmic, and the addition of an SV40 NLS redirects the protein to the nucleus. HeLa cells were transfected with vectors expressing either mt-p21, mt-p21(1–141), or mt-p21(1–141)-NLS stained with anti-myc-tagged antibody.

(D) NIH3T3 cells were transfected with either CS2p21, CS2p21(1–141), or CS2p21(1–141)-NLS and treated with Mg-132 as indicated.

retargeted p21 (1–141) to the nucleus, and the protein regained its proteasome sensitivity.

In these respects, the turnover of p21 differs markedly from its close relative p27Kip1, which has been shown to be degraded in the cytoplasm. Furthermore, the nuclear export of transfected p27 is blocked by both proteasome inhibitors and the nuclear export inhibitor leptomycin B, thereby sequestering p27 in a cellular compartment where it is relatively stable (the nucleus) (Tomoda et al., 1999). In contrast, we find that p21 is degraded in the nucleus but is relatively stable in the cytoplasm. Furthermore, we have not observed any effect of leptomycin B on the abundance of transfected p21 (data not shown). Thus, unlike p27, proteasome inhibition does not indirectly stabilize p21 by restricting it to a cellular compartment where it is stable.

Discussion

p21 is an unstable protein exhibiting proteasome-sensitive turnover and ubiquitination in vivo. However, we have shown that p21 remains unstable and proteasome dependent even when it cannot be ubiquitinated. Therefore, direct p21–ubiquitin conjugates are not obligatory intermediates in proteasome-dependent p21 turnover. These data do not exclude the possibility that p21 turnover may be mediated by ubiquitination in some physiologic contexts. Alternatively, p21 ubiquitination may serve some function other than signaling proteolysis. Regardless, the example of p21 illustrates that observing multiubiquitination and proteasome sensitivity in vivo are insufficient to conclude that protein turnover must proceed through a ubiquitinated intermediate.

How might p21 be degraded by the proteasome independently of ubiquitin attachment? The clearest example of a protein whose turnover by the proteasome is ubiquitin independent is ornithine decarboxylase (ODC), and additional examples have been proposed (Murakami et al., 1992; Jariel-Encontre et al., 1995; Yu et al., 1997). ODC is directed to the proteasome by its specific binding partner, antizyme. Similarly, the interaction of p21 with its known binding partners affects its turnover, although these relationships are complex and poorly understood (Cayrol and Ducommun, 1998; Rousseau et al., 1999; unpublished observations). Perhaps the simplest explanation is that nonubiquitinated p21 is directly recognized by the proteasome. Unstructured proteins can be directly recognized and degraded by proteasomes in vitro and in vivo without ubiquitination (Katz-nelson and Kulka, 1985; Wenzel and Baumeister, 1993; Michalek et al., 1996). Moreover, free p21 does not have a well-defined tertiary structure (Kriwacki et al., 1996, 1997). Thus, p21 and perhaps other small Cdk inhibitors might be directly recognized by the proteasome when they are free of cyclin/Cdk complexes and adopt unstructured conformations.

The presence of ubiquitinated p21 suggests that p21 is associated with ubiquitinating enzymes. An interesting possibility is that p21 may be recruited to the proteasome by being bound to a protein that is itself ubiquitinated. Components of the SCF, an E3 ubiquitin ligase that regulates the abundance of G1 cyclins and Cdk

inhibitors in yeast, are themselves ubiquitinated (Zhou and Howley, 1998; Galan and Peter, 1999), and mammalian homologs of SCF proteins have been identified (Peters, 1998; Koepp et al., 1999). In fact, p21 has been reported to physically interact with complexes containing SCF proteins, and antisense inhibition of SCF proteins leads to p21 overaccumulation in vivo (Yu et al., 1998; Yam et al., 1999). Thus, perhaps p21 is recruited to the proteasome through its physical association with a ubiquitinated E3 complex.

Finally, we cannot exclude the possibility that proteasome inhibitors affect p21 turnover indirectly. For instance, proteasome inhibition may indirectly stabilize p21 by affecting protein(s) that regulate p21 abundance. Thus, p21 might be stable when bound to a protein that is itself proteasome dependent, or a p21 protease may be regulated by the proteasome.

Defining the role of the ubiquitin–proteasome system in protein turnover is complicated by its involvement in diverse biological processes. In the case of p21, our data support the notion that p21 turnover is regulated by the proteasome but indicate that this does not require direct p21 ubiquitination. The relative importance of direct ubiquitination in the degradation of other proteins, particularly mammalian proteins that have been analyzed only in vivo, remains a difficult issue. In some cases, the role of specific lysine residues has been directly demonstrated (examples include Scherer et al., 1995; Baldi et al., 1996; King et al., 1996b; Rodriguez et al., 1996; Yu et al., 1997). More commonly, the relative importance of direct ubiquitination in proteasomal protein degradation has not yet been determined.

Experimental Procedures

Cell Culture and Transfections

Human diploid fibroblasts were provided by C. Grandori (Seattle, WA); NIH3T3 and 293 cells were previously described (Sheaff et al., 1997). All cell lines were grown and transfected as described previously (Clurman et al., 1996). In most experiments, DNA concentration was 6 μg/60 mm dish. Different experimental conditions are noted. For wt-p21, p21-K6R, and p21 (1–141), NIH 3T3 cells and 293 cells were transfected with 2 μg and 1 μg of expression vector/60 mm dish, respectively, whereas 250 ng/60mm dish of mt-p21, mt-p21K6R, and HA-p21 vectors was used.

Plasmids, In Vitro Mutagenesis, and Retroviral Vectors

Plasmids were obtained from: pHA-ubiquitin (M. Treier, Heidelberg, Germany); p-CMV-CDK2 (E. Harlow, Charlestown, MA); pUBR7 (T. McGarry, R. King, and M. Kirschner, Boston, MA); pUNI15, pHM200-myc3, and pHM200-HA3 (S. Elledge, Houston, TX); and pLNSX (D. Miller, Seattle, WA). pCS2hp21, pCS2mt-cyclin E (M130A), pCS2mt-cyclin E, and pCS2mt-cyclinA were previously described (Clurman et al., 1996). CS2-p21K6R and CS2-MTEΔK were constructed from pCS2p21 and pCS2m-cyclin E by converting all of the lysine residues in each protein to arginine as described (Clurman et al., 1996). Primer sequences are available upon request.

For LNSX-p21 and LNSXp21K6R viral stocks, the CS2 p21 and p21K6R inserts were subcloned into the retroviral vector pLNSX (Miller and Rosman, 1989). Retroviral supernatants were prepared by transfecting the producer line Phoenix-Eco (obtained from G. Nolan, Palo Alto, CA.) with pLNSX-p21 and pLNSX-p21K6R and harvesting supernatants after 48 hr. 500,000 NIH3T3 cells were transduced with 3 ml of viral supernatant and selected with G418 (3 mg/ml).

The HA3-p21, HA3p21K6R, mt3p21, and mt3p21K6R constructs were made with the Univector system as described (Liu et al., 1998). The cDNAs of human p21 and p21K6R were subcloned into pUNI15 and recombined with either pHM200-myc3 or pHM200-HA3. p21(1–141) and p21(1–141NLS) were produced by generating PCR fragments beginning with the p21 ATG and ending at either residue 141, or adding KKKRKV (corresponding to the SV40 NLS) after residue 141, and subcloned into pCS2 or pCS2MT (myc-tagged) and sequenced.

Antibodies

The following antibodies were used: monoclonal anti-p21 (Transduction Labs); polyclonal anti-p21 antibody (C19), monoclonal anti-p21 antibody (F5), and anti-Cdk2(M2), (Santa Cruz Biotechnology); monoclonal anti-human cyclin E HE12 (Pharmingen), HRP-conjugated anti-mouse and anti-rabbit IgG (Amersham), FITC-anti mouse IgG, or anti-rabbit IgG (Jackson Labs). 9E10 (anti-myc tag) was prepared by an in-house facility.

Polyclonal anti-p21(C-19) detects both mouse and human p21 and was used to directly compare the expression of retrovirally expressed wild-type human p21 and endogenous mouse p21. Because the epitope recognized by this antibody contains lysines, it detects p21K6R poorly (see Figure 3A). Monoclonal p21 (Transduction Labs), which recognizes only human p21, was thus used to compare the level of wt-p21 to p21K6R expression and allows an indirect comparison of p21K6R and endogenous p21 expression.

Inhibitors

LLM (a control peptide aldehyde that does not inhibit the proteasome), Mg-132 (Calbiochem), and β-lactone (Boston Biochemical) were dissolved in DMSO. Approximately 24 hr after transfection, cells were treated with inhibitors overnight (LLM, 50 μM; MG-132, 2 μM; β-lactone, 10 μM). Leptomycin B was a gift of M. Yoshida (Tokyo) and used at 10 ng/ml.

Western Blotting, Immunoprecipitation, and Kinase Assays

Cells were lysed in RIPA buffer (10 mM tris [pH 7.4], 0.15 M NaCl, 1% NP-40, 1% deoxycholate, 0.1% SDS, 10 μg/ml each of aprotonin, leupeptin, and pepstatin, 50 mM NaF, 1 mM Na vanadate), followed by scraping and sonication. Lysates for coimmunoprecipitation were made in NP-40 buffer (0.5% NP-40, 20 mM Tris [pH 7.4], 150 mM NaCl). Cell extracts were electrophoresed on 12%–17% polyacrylamide gels and Western blotted was as described (Clurman et al., 1996). For immunoprecipitation, cell lysates normalized for protein concentration were processed as described (Clurman et al., 1996). For the p21 kinase assay, after the last wash, immunoprecipitates were resuspended in 20 μl kinase buffer containing 1 μm ATP + 0.5 μl [γ-^{32}P]ATP (6000 Ci/mM, NEN) and incubated at 37°C for 30 min.

Pulse–Chase

For endogenous p21, cells in 100 mm dishes were labeled with Tran^{35}label (ICN) at 500 μCi/ml in 2.2 ml DME without methionine/cysteine (ICN) for 15 min at 37°, then chased with DME/10% FBS with 400 mg/L methionine. Cells were lysed in RIPA and immunoprecipitated with polyclonal anti-p21 antibody (C-19) after preclearing. Modifications for transfected p21 were as follows: first, cells were transfected on 70%–80% confluent 100 mm dishes, and each dish was split into 6–8 60 mm dishes so that each time course was derived from the same transfection; second, 200 μCi/ml of label was used, and half of each 60 mm plate lysate immunoprecipitated with monoclonal anti-p21 (Transduction Labs). For pulse–chase analyses after proteasome inhibition, cells were treated with MG-132 or β-lactone for 2 hr prior to labeling, and inhibitors were present throughout all subsequent stages. Pulse–chase analyses of NIH-LNSXp21 and NIH-LNSXp21K6R were performed as described for endogenous p21, and lysates immunoprecipated with monoclonal anti-p21 (Transduction Labs) or monoclonal anti-p21 F5 (Santa Cruz).

Indirect Immunofluoresence

NIH3T3 or HeLa cells were transfected as above with glass coverslips in the dish. Cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100/PBS, then stained with primary antibody followed by FITC-anti-mouse IgG or FITC-anti-rabbit IgG (Jackson Labs). The final wash contained DAPI to visualize nuclei.

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